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Isolation of *Salmonella enterica* Serovar Enteritidis from Houseflies (*Musca domestica*) Found in Rooms Containing *Salmonella* Serovar Enteritidis-Challenged Hens[▽]

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Houseflies (*Musca domestica*) released into rooms containing hens challenged with *Salmonella enterica* serovar Enteritidis (*Salmonella* serovar Enteritidis) rapidly became contaminated with *Salmonella* serovar Enteritidis. Forty to 50% of the flies were contaminated at 48 h, and the percentage increased to 50 to 70% at 4 and 7 days postexposure and then decreased to 30% at day 15. Initial attempts at recovering surface organisms for culture using an aqueous rinse were largely unsuccessful, while cultures of internal contents readily recovered *Salmonella* serovar Enteritidis. However, when 0.5% detergent was incorporated into the rinse, high recovery levels of bacteria were observed from both external and internal culture regimens, indicating equal distribution of the organism on and in the fly and a tighter interaction of the organism with the host than previously thought. *Salmonella* serovar Enteritidis was isolated routinely from the fly gut, on rare occasions from the crop, and never from the salivary gland. Feeding contaminated flies to hens resulted in gut colonization of a third of the birds, but release of contaminated flies in a room containing previously unchallenged hens failed to result in colonization of any of the subject birds. These results indicate that flies exposed to an environment containing *Salmonella* serovar Enteritidis can become colonized with the organism and might serve as a source for transmission of *Salmonella* serovar Enteritidis within a flock situation.

Salmonella enterica serovar Enteritidis (*Salmonella* serovar Enteritidis) remains a serious food-borne threat to humans within the U.S. and overseas (6, 30). Poultry and their products constitute a significant proportion of the sources implicated in food-borne *Salmonella* outbreaks (30, 34, 36), prompting more rigorous focus by regulatory agencies and industry on implementing measures to reduce the incidence of these problem organisms on the farm and during processing. Many risk factors for exacerbating *Salmonella* infection in flocks have been identified on the farm, and animal vectors, both vertebrate and invertebrate, have been implicated in this role (13, 14, 37). Insects have long been associated with the spread of pathogens in human disease outbreaks (1, 9), and similar observations have been reported for poultry. Cockroaches (24), beetles (25, 33), and flies (10, 25, 29) recovered from poultry houses have all been reported to harbor *Salmonella* or other human pathogens, and chicken-to-chicken transmission of these organisms has also been observed (32).

Flies comprise a large and complex fauna of arthropods with worldwide distribution. Because of their intimate relationship with decaying matter, garbage, and feces, flies have long been associated with the potential for spreading disease. A. R. Olsen (28) lists 47 fly species from which *Escherichia coli*, an indicator of fecal contamination, have been isolated. Seventeen of these were found to carry the human pathogens *Salmonella* or *Shigella* and, of these, 14 were considered "communicative," meaning that they moved between contaminated environments and interacted with man (8, 28). The housefly (*Musca domestica*) was a prominent member of this group and received the most citations regarding contamination with human pathogens. With regard to poultry, while a number of studies examined flies as carriers of *Salmonella*, no work has focused on the kinetics of colonization of flies with *Salmonella* upon exposure to a contaminated environment or where this organism resides on or in its arthropod host. The current study examined the time frame for fly contamination upon release into a room containing hens infected with *Salmonella* serovar Enteritidis, the location of the organism on or in the fly, and whether these contaminated flies could transmit *Salmonella* to naive nonstressed and stressed hens.

MATERIALS AND METHODS

Chickens. Single-comb white leghorn chickens >60 weeks of age were obtained from the specific-pathogen-free flock maintained at the Southeast Poultry Research Laboratory (SEPRL), Athens, GA. Twenty-six, 24, and 26 hens were used in experiments 1, 2, and 3, respectively. The hens were transferred to individual, adjacent laying cages in an environmentally controlled biosafety level 2 building at SEPRL and allowed to acclimate for 7 to 4 days. The hens were fed layer rations ad libitum throughout the duration of the experiment. To ensure that the hens were *Salmonella*-free, the individuals were screened for *Salmonella* prior to the commencement of the experiments by enriching 1 g of feces in 9 ml of Rappaport-Vassiliadis (RV) enrichment medium (Oxoid, Inc., Basingstoke, England), which was incubated overnight at 37°C, after which 100 μl of the broth was plated onto XLT4 agar (Remel, Lenexa, KS). *Salmonella* was not detected. The studies were approved by and conducted under the guidelines of the SEPRL Institutional Animal Care and Use Committee.

Infection. Frozen stocks of nalidixic acid-resistant *Salmonella* serovar Enteritidis (strain SE89-8312) were maintained at −20°C. For each experiment, 3 days prior to infection, *Salmonella* serovar Enteritidis cells were thawed and cultured onto nutrient agar (Difco/Becton Dickinson Microbiology Systems, Sparks,

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Maryland) at 37°C for 18 to 24 h. An individual colony of *Salmonella* serovar Enteritidis was recultured onto nutrient agar and incubated at 37°C for 18 to 24 h. A 1- ml tube of tryptic soy broth (Difco) was then inoculated with isolated colonies from the nutrient agar plate and incubated overnight at 37°C. The *Salmonella* serovar Enteritidis broth culture was diluted to 10^{-2} cells in sterile saline, and each bird received a dose of 1 ml per os (9×10^6, 5.6×10^6, and 3×10^6 *Salmonella* serovar Enteritidis cells in experiments 1, 2, and 3, respectively). At the time of challenge, six food-grade 23-by-28-cm polystyrene trays (Genpack Corp., Glens Falls, NY) were placed on the floor beneath the cages in each room and served as the sites for sampling environmental fecal levels of *Salmonella* serovar Enteritidis contamination. On testing days, four 1-g fecal samples were obtained from individual trays and placed into separate plastic stomacher bags. The sites were randomly selected each day. The samples were diluted 10-fold in RV broth and then plated onto brilliant green agar containing 20 μg/ml of nalidixic acid and novobiocin (BGNN), using an Autoplate 4000 automatic dilution/plating system (Spiral Biotech, Norwood, MA). After 24 h of incubation at 37°C, counts of *Salmonella* serovar Enteritidis cells on the plates were made, using a QCount plate reader (Spiral Biotech). For the samples with no detectable growth of *Salmonella* serovar Enteritidis on the plates, the RV enrichment medium for that sample was plated onto BGNN. These plates were incubated for a further 24 h at 37°C and evaluated for the presence of *Salmonella* serovar Enteritidis.

Flies and sampling. Houseflies (*Musca domestica*) were received as pupae, approximately 2 days prior to emergence as flies, from the USDA/ARS Center for Medical and Veterinary Entomology, Gainesville, FL. Approximately 10^4 pupae were received in a cardboard container, and the container, with the lid removed, was placed in the bird room. The placement occurred 3 days prior to hen challenge to ensure maximum fly density at the time the birds received the *Salmonella* serovar Enteritidis. A second batch of 10^4 pupae was placed in the rooms 7 days later to replenish the fly population. In order to encourage natural visitation by the flies to the birds and the food and feces, no additional food was provided to the flies. On sampling days, one QuikStrike fly abatement strip (Wellmark International, Schaumburg, IL) was placed on a fresh polystyrene tray on the floor of each room. The flies attracted to the bait on the strip consumed a portion of it and rapidly succumbed to the nithiazine toxicant. The dead flies were retrieved, using sterile forceps, one forceps per fly, and deposited into individual zip-sealable plastic bags. The flies were collected individually immediately after knockdown to eliminate cross-contamination after knockdown. Twenty flies per room were collected on each sampling day and transported to the laboratory for culturing. Each bag received 1 ml of RV broth, and the flies were thoroughly crushed and macerated into the broth, using the thumb and forefinger. One hundred microliters of the sample was then spread-plated onto BGNN. The plates and the bags containing the remainder of the fly-RV mixture were incubated for 24 h at 37°C. Following incubation, the BGNN plates were evaluated and *Salmonella* serovar Enteritidis cell counts were ascertained. For the samples with no detectable *Salmonella* serovar Enteritidis growth on the plates, the RV enrichment for that sample was plated onto BGNN, and the plates were incubated a further 24 h at 37°C and evaluated for the presence of *Salmonella* serovar Enteritidis. Samples with no growth on the direct plating but which tested positive in the RV enrichment medium were given an arbitrary count of 9 (1 point below the theoretical detection limit), and samples with no growth in either the direct plating or the RV enrichment medium were given a count of 0.

Localization of *Salmonella* serovar Enteritidis cells on or in the fly. Three trials were performed to determine the location of *Salmonella* serovar Enteritidis cells on or in the fly. In trial 1, seven flies were collected from experiment 2 hens into individual sterile tubes on each of 2 separate days. One milliliter of RV broth was added to each tube, and the tubes were vortexed at maximum speed for 30 s (rinsed). Each fly was then placed into a tube containing 1 ml of bleach (800 ppm chlorine), which was vortexed for 30 s and then placed into a tube containing 1 ml of sterile distilled water and vortexed for a further 30 s. The fly was removed from the water and added to a tube containing 1 ml of RV broth and vortexed for a further 30 s (cleaned). The flies were then transferred individually to 1 ml of fresh RV broth contained in a zip-sealable plastic bag and crushed and macerated between the thumb and forefinger (inside). All samples were incubated at 37°C for 24 h and then plated onto BGNN. Following a further 24-h incubation at 37°C, the plates were examined for the presence of *Salmonella* serovar Enteritidis. The results from the 2 days were combined, and statistics were run on the combined results. In trial 2, 10 flies were collected from the room containing experiment 3 hens, day 3 postchallenge, and placed into a tube containing 1 ml of phosphate-buffered saline (PBS)–0.5% Tween 20 (Sigma Chemical Co., St. Louis, MO). The tube was vortexed for 30 s, and the PBS–0.5% Tween20 was transferred to 9 ml of RV broth (exterior wash). The fly carcass was then added to 1 ml of bleach (1,000 ppm), vortexed for 30 s, and allowed to sit for 14.5 min. The salivary gland, crop, and gut posterior from the proventriculus from each fly were aseptically excised and placed en mass into 5 ml of RV broth. All samples were incubated for 24 h at 37°C and then plated onto BGNN, and the plates were incubated for a further 24 h at 37°C and examined for the presence of *Salmonella* serovar Enteritidis. In trial 3, 10 flies were collected from the room containing experiment 3 hens, day 3 postchallenge, and frozen for 24 h. The individual flies were thawed and placed into bleach (1,000 ppm) for 15 min and then dipped into PBS to remove the bleach. The salivary gland, the crop, and then the gut were aseptically excised from each fly and placed into a separate 5-ml RV broth for each tissue. The broths were incubated at 37°C for 24 h and then plated onto BGNN, incubated a further 24 h at 37°C, and examined for the presence of *Salmonella* serovar Enteritidis.

Transmission of *Salmonella* serovar Enteritidis via contaminated flies. Two trials were conducted to examine the transmission of *Salmonella* serovar Enteritidis to previously unchallenged hens. In trial 1, 200 flies were captured live from experiment 2 hens at day 7 postchallenge and released into a room containing 24 hens from which feed had been removed 3 days previously, a time point chosen because of previous observations of the time of maximal immunosuppression (15). Intestinal shedding of *Salmonella* serovar Enteritidis by the hens was examined on days 2 and 9 following release of the flies by administering 0.5 ml of 0.5% pilocarpine solution (Sigma) intraperitoneally to each hen and collecting the intestinal secretions released over the next hour onto polystyrene trays placed directly beneath each cage (21). One milliliter of the secretion was added to 9 ml of RV broth. Following 24 h of incubation at 37°C, the broths were plated onto BGNN and were examined for the presence of *Salmonella* serovar Enteritidis 24 h later. In trial 2, 16 hens (8 hens/room) were transferred to two separate rooms and allowed 7 days to acclimate. Feed was removed for 14 days from the birds in one room, and on day 3 after the removal of feed, the hens in both rooms received five flies per bird, administered with a Pipetteman P1000 (Rainen Instruments, Woburn, MA). The flies, retrieved on day 9 of experiment 3, were loaded five flies per tip into P1000 tips whose ends were clipped to widen the bore diameter and allow passage of the flies. The tips were inserted onto the pipettor, and the flies were expelled down the esophagus of each hen. The hens were sampled for the presence of *Salmonella* serovar Enteritidis in the crop and intestinal tract. Feed was removed from the fed group of birds the night before the sampling. Intestinal shedding was determined by intraperitoneal pilocarpine administration as described above. Crop colonization was determined as described previously (22). Briefly, 20 cm of tubing (Tygon; interior diameter, 1/8; outer diameter, 3/16; Fisher Scientific) attached to a 10-ml syringe was inserted down the esophagus into the crop, and 5 ml of crop lavage solution (1 M Tris-glycine buffer with 0.25% Tween 20 [pH 7 to 8]) was administered into the crop. The lavage solution, along with the luminal contents of the crop, was aspirated immediately back into the syringe and aseptically transferred into a 15-ml conical tube. Undiluted intestinal and crop samples (100 μl) were spread-plated onto BGNN plates, and 1 ml of each undiluted sample was added to 9 ml of tetrathionate brilliant green broth. The intestinal samples were also diluted 1:10 and plated onto BGNN plates, using an Autoplate 4000 automatic dilution/plating system. The plates and broth enrichments were incubated for 24 h at 37°C, and the plates were examined for growth of *Salmonella* serovar Enteritidis. For any sample negative for *Salmonella* serovar Enteritidis, the tetrathionate enrichment broths were plated onto BGNN and examined for the presence of *Salmonella* serovar Enteritidis cells the next day. Samples with no growth on the direct plating but which were positive in the tetrathionate enrichment broth were given an arbitrary count of 9 (1 point below the theoretical detection limit), and samples with no growth in either the direct plating or the tetrathionate enrichment broth were given a count of 0.

Statistical analyses. Statistical analyses were performed using GraphPad Instat (GraphPad Software, Inc., San Diego, CA). Unpaired *t* tests were performed on comparisons between the numbers of *Salmonella* serovar Enteritidis cells recovered from fly exteriors and interiors in trial 1 and comparisons of chicken crop versus intestinal colonization by the organism following administration of contaminated flies. Analysis of variance with Tukey's multiple comparison test procedures was conducted to compare the numbers of *Salmonella* serovar Enteritidis cells recovered from the different organs within the fly (trial 3).

RESULTS

Fly contamination. The densities of flies in the rooms at the time the hens were challenged approached 200 flies/m^3, which would be considered a low-to-moderate number in commercial egg operations (J. J. Arends, North Carolina State University

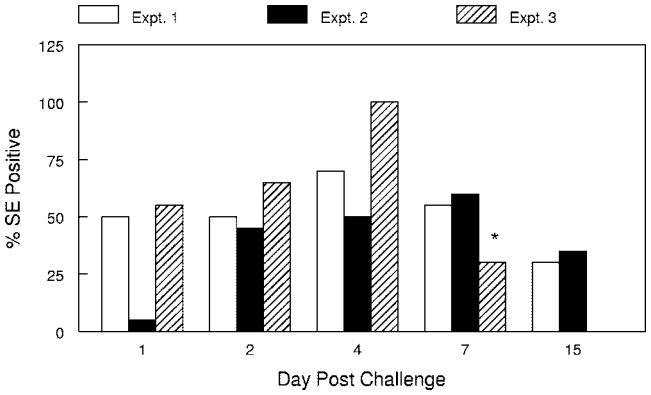

FIG. 1. Recovery of *Salmonella* serovar Enteritidis (SE) from flies captured in rooms containing infected hens. The results represent the percentage of flies (n = 20 flies/sampling day) that were positive for *Salmonella* serovar Enteritidis on each sampling day postchallenge in experiments 1, 2, and 3. Expt., experiment; *, flies sampled on day 9 postchallenge in experiment 3.

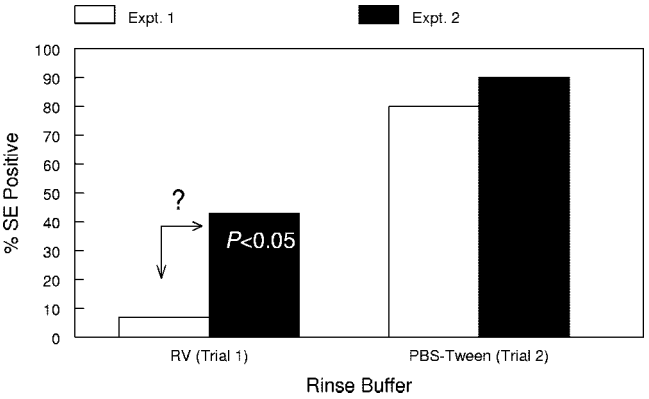

FIG. 3. Simple aqueous rinsing does not remove *Salmonella* serovar Enteritidis (SE) from fly exteriors. The results represent the percentage of flies that were positive for *Salmonella* serovar Enteritidis for each sample type in trial 1 (n = 14 flies/sample type) and trial 2 (n = 10 flies/sample type). The number within the bar is the statistical difference between the numbers of *Salmonella* serovar Enteritidis cells recovered from fly interiors and exteriors. ?, questionable lack of organism.

Department of Entomology, personal communication). Within 48 h, 45 to 50% of the flies in experiments 1 and 2 possessed detectable amounts of *Salmonella* serovar Enteritidis cells, and recovery of the organisms remained at 50% or greater for the next 5 days (Fig. 1). The numbers of *Salmonella* serovar Enteritidis cells residing in or on the flies ranged from 9 to 10^4. In experiment 3, 55% of the flies were positive for *Salmonella* serovar Enteritidis 24 h postchallenge, and the percentage increased to 100% by day 4 and decreased thereafter (Fig. 1).

Fecal contamination with *Salmonella* serovar Enteritidis. Considerable fecal matter accumulated on the floors beneath the cages over time. Levels of *Salmonella* serovar Enteritidis ranged from 10^3 to 10^7 cells/g feces the first week postchallenge and decreased to 10^3 to 10^5 cells/g by the end of the second week in both experiments (Fig. 2).

Localization of *Salmonella* serovar Enteritidis on or in the fly. In trial 1, only a small percentage of flies possessed any detectable amount of exterior *Salmonella* serovar Enteritidis cells, as indicated by culture results from the RV wash of each fly carcass. Conversely, a higher percentage ($P < 0.05$) of the fly interiors contained the organism (Fig. 3). Repeat experiments using an RV wash or PBS showed similar results. This surprising observation called into question the efficacy of using a simple aqueous wash to break the interaction between the *Salmonella* serovar Enteritidis cells and the fly. Using a PBS wash solution supplemented with 0.5% Tween 20, a nonionic detergent, resulted in a dramatic increase in the numbers of *Salmonella* serovar Enteritidis cells recovered from the fly exteriors (Fig. 3), equaling the recovery rate obtained from culturing the fly interior, 80 to 90%. In an effort to determine where within the fly the *Salmonella* serovar Enteritidis cells resided, salivary glands, crops, and guts were individually cultured; the results are shown in Table 1. No *Salmonella* serovar

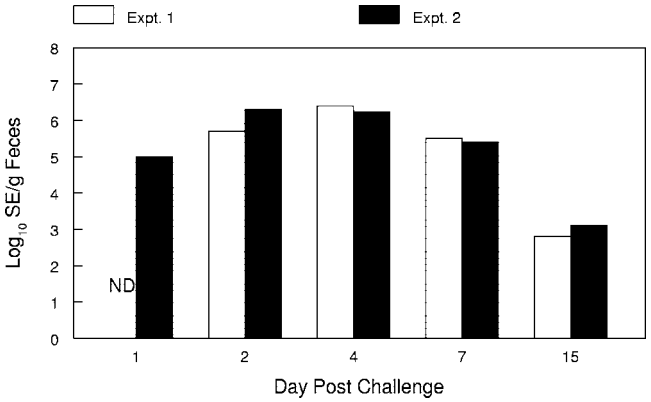

FIG. 2. Concentrations of *Salmonella* serovar Enteritidis (SE) in feces that collected under cages of infected hens over different times postchallenge (n = 4 samples/time period). The results represent the $\log_{10}$ *Salmonella* serovar Enteritidis cells/gram of feces at the sampling times noted for experiment 1 (Expt. 1) and experiment 2 (Expt. 2). ND, not determined.

TABLE 1. Locations of *Salmonella* serovar Enteritidis within the digestive tracts of flies retrieved from rooms contaminated with *Salmonella* serovar Enteritidis[a]

Fly no.	Salivary gland[b]	Crop[b]	Gut[b]
1	−	−	+
2	−	−	+
3	−	−	+
4	−	−	+
5	−	−	+
6	−	−	+
7	−	−	+
8	−	−	+
9	−	−	+
10	−	+	+
No. positive/total	0/10	1/10	10/10[c]

[a] The results are for individual flies recovered from rooms housing hens challenged with *Salmonella* serovar Enteritidis on day 3 postchallenge.

[b] The salivary gland, crop, and then the gut were aseptically dissected out from exteriorly disinfected individual flies and cultured. The results represent the presence (+) or absence (−) of *Salmonella* serovar Enteritidis cells in each tissue from each fly.

[c] $P < 0.001$ versus results for the crop and salivary gland.

TABLE 2. Initiation of *Salmonella* serovar Enteritidis infection in fasted versus nonfasted hens by oral administration of flies contaminated with *Salmonella* serovar Enteritidis[a]

Day postchallenge	% of indicated tissues from fed hens positive for *S.* serovar Enteritidis		*P* value[b]	% of indicated tissues from fasted hens positive for *S.* serovar Enteritidis		*P* value[b]
	Crop	Intestine		Crop	Intestine	
6	0	38	<0.05	13	38	NS
12	0	25	NS	13	29	NS
20	0	13	NS	0	14	NS

[a] Fed and fasted hens were orally administered flies contaminated with *Salmonella* serovar Enteritidis, and the presence of the organism in crop and fecal samples was monitored over time.
[b] *P* values were determined from results for the crop versus those for the intestines. NS, not significant.

Enteritidis cells could be detected in fly salivary glands, and only 10 to 20% of the fly crops had detectable amounts of the organism. *Salmonella* serovar Enteritidis cells were recovered from all of the fly guts ($P < 0.001$ versus the numbers for the salivary gland and crop).

Transmission of *S. enteritidis* via contaminated flies. To determine whether flies possessing *Salmonella* serovar Enteritidis could transmit the organism to naive hens, a study was conducted in which contaminated flies were released into a room containing fasted naive hens. Infection of the hens by *Salmonella* serovar Enteritidis via exposure to the contaminated flies was not observed (data not shown). To determine whether *Salmonella* serovar Enteritidis from contaminated flies would be capable of colonizing hens, a second study was conducted in which the birds were administered contaminated flies per os and then monitored for shedding of the organism over the next 3 weeks. An additional parameter, feed withdrawal, was assigned for one group, as this was shown previously to dramatically increase susceptibility to infection (17). As shown in Table 2, minimal colonization of the chicken crop could be observed, while intestinal colonization occurred in approximately 38% of the birds at days 6 ($P < 0.05$ versus 0% in the crop samples from fed hens) and 13 postchallenge and dropped to 13 to 14% at day 20.

DISCUSSION

Flies from environments contaminated with human pathogens readily become contaminated themselves. Rosef and Kapperud showed in 1983 that 51% and 43% of flies captured on chicken farms and piggeries, respectively, in Norway were positive for *Campylobacter* (31). Bailey et al., were able to isolate *Salmonella* from 19% of flies captured on broiler farms (2), while Olsen and Hammack (29) found a 22% carrier rate of *Salmonella*, including *Salmonella* serovar Enteritidis, in flies captured in facilities housing laying hens. Numerous other studies have reported similar results (10, 27, 37). To the authors' knowledge, no studies have followed the kinetics of colonization with human pathogens of flies exposed to infected animals and their environments. Greenberg et al. (9) fed gnotobiotic houseflies graded doses of *S. enterica* serovar Typhimurium and found that the flies excreted the organism in their feces at 24 h postchallenge. The percentage of *Salmonella* excretors was dramatically reduced by 96 h postchallenge, indicating that, for the flies to remain positive for *Salmonella* over time, they needed a reexposure. Shane et al. (32) showed that houseflies housed within an isolator cabinet containing chickens fecally shedding *Campylobacter jejuni* became colonized with the organism at 5 days. The current study demonstrated that the common housefly can rapidly become contaminated with *Salmonella* serovar Enteritidis by residing in an environment housing hens challenged with that organism. This contamination occurred within 24 to 48 h postchallenge of the hens and persisted for at least 2 weeks. Greenberg et al. (9) showed that a reexposure was necessary for *Salmonella* to persist in the fly host, and a similar reexposure to the contaminated fecal matter was probably necessary to allow the long-term prevalence of *Salmonella* serovar Enteritidis.

The initial study comparing the frequency of recovery of *Salmonella* serovar Enteritidis from the fly exterior versus the interior indicated that the organism could be much more readily recovered from the fly interior and that the frequency of recovery from the exterior was very low (Fig. 3). This finding was observed on repeat occasions (data not shown) and is counter to the general consensus regarding flies as vectors of human pathogens via a transfer of the organism through simple contact. Insects possess a waxy cuticle which covers their entire exteriors and serves as a barrier to water loss and also renders them waterproof (26). Adding a lipid solvent such as a detergent dissolves the wax and eliminates the waterproof character of the insect. Indeed, when the detergent Tween 20 was added to the fly rinse buffer in the current study, recovery of *Salmonella* serovar Enteritidis cells from the fly exterior increased to levels matching that observed for the interior (Fig. 3). These results indicate that the interaction of *Salmonella* serovar Enteritidis with the fly exterior is more dynamic than simply sitting on the surface of the fly. Further, simple physical contact may not be the primary method of transfer of the organism to different surfaces in a house, and other mechanisms may be more prevalent.

Different microbial pathogens can reside in the fly alimentary tract (4, 7, 9, 12, 32). Greenberg et al. (9) showed that *Salmonella* serovar Typhimurium fed to flies will reside and multiply in the fly gut. A high percentage of gut samples were culture-positive for *Salmonella* serovar Typhimurium, while minimal recovery of *Salmonella* serovar Typhimurium cells from the crop was observed. The results shown in Table 1 of the present study mirror those of Greenberg et al. in that a high percentage, 100%, of the gut samples were positive for *Salmonella* serovar Enteritidis, while only 15% of the crops had detectable amounts of *Salmonella* serovar Enteritidis (compared with a previously reported 89% and 11%, respectively, for gut and crop samples [Greenberg et al.] [9]). These results indicate that crop regurgitation by the fly during food collection is probably not a major source of pathogen spread, while defecation by the fly is a much better possibility.

The importance of the carriage of different pathogens by flies lies in their ability to transmit the pathogen to susceptible hosts. In 1985, Shane et al. (32) showed that houseflies taken from isolation cabinets housing *Campylobacter*-infected chickens shedding the bacterium fecally and introduced into a second isolation cabinet containing naive chickens could transmit

Campylobacter to these recipient birds. In the current study, transmission of *Salmonella* serovar Enteritidis was attempted through the introduction of flies collected from rooms containing infected hens into a room containing naive hens. To increase the potential for transmission to occur, feed was withdrawn from the recipients, mimicking a layer industry management tool, induced molting, used to achieve multiple egg-laying cycles from aging flocks. Prior to January 2006, withdrawal of feed until the flock dropped 30% in body weight, 10 to 14 days, was the primary method for flock recycling. This methodology has since been replaced in the U.S. with less-stressful diet regimens (35), while in Latin America and the Far East, responsible for more than 50% of egg production worldwide, feed withdrawal remains the primary method for molting flocks. Earlier studies have shown that feed withdrawal depresses cellular immunity in the birds (15, 16), resulting in an increased severity of infection by *Salmonella* serovar Enteritidis (17, 18, 19, 20, 21) and dramatically increasing the susceptibility of hens to infection (17). In the present study, this increased susceptibility had no apparent impact on the transmission of *Salmonella* serovar Enteritidis to the recipient birds, as no birds were found to be colonized by the organism. There are several possible explanations for the discrepancy. *Campylobacter* and *S. enteritidis* are entirely different organisms, and their transmission capabilities may therefore differ. Also, while birds in both studies received equivalent numbers of flies, the birds in the earlier study were housed in a relatively small, contained space, a Horsfall isolation cabinet (0.6 m by 0.6 m by 0.6 m), while the hens in the present study were housed in a 5 m by 3 m by 3 m animal room. The latter situation dramatically expanded the space that the flies could traverse, eliminated the intimate interaction brought on by small, enclosed spaces, and decreased the fly density from 1,000 flies/m^3 to 5 flies/m^3. However, *Salmonella* serovar Enteritidis-contaminated flies can transmit the organism when they are fed to naive hens (Table 2), indicating that transmission of *Salmonella* serovar Enteritidis by introducing contaminated flies into a room containing naive hens may be possible if a larger number of contaminated flies were used in the study or, conversely, by housing the recipient birds in isolation cabinets to enhance the contact between fly and bird. Note that the number of flies released in this study was relatively low and represented a density substantially below that commonly observed in commercial flocks.

The chicken crop is an enlargement or outpouching of the esophagus just proximal to the proventriculus, or glandular stomach. The primary function of the crop is food storage while the stomach is full. The chicken crop was shown to routinely harbor *Salmonella* and was touted as an important source of broiler carcass contamination during processing (3, 5, 11). Birds challenged with *Salmonella* serovar Enteritidis showed equal levels of colonization of the crop and intestinal tract (23). Interestingly, few of the crops from the recipient hens were culture positive for *Salmonella* serovar Enteritidis. Why these birds differed from those in previous studies remains to be determined. It may simply be a function of bacterial density, in that most challenge studies use 10^4- to 10^6-cell challenge doses, while the flies in the current study generally carried many fewer organisms (range, 9 to 10^4 organisms). Further, the crop tissues were bathed in the organism following oral challenge, while the fly-derived *Salmonella* would be trapped in and on the fly and just transit on through the organ. In the field broiler (5, 11) studies, the birds spent their life spans on contaminated poultry litter and, as a certain degree of coprophagy occurs during broiler grow-out, the crop was regularly recontaminated and recolonized.

The present study reinforces what has been known for a long time, that flies residing in a contaminated environment will, themselves, become contaminated. However, to the authors' knowledge, the rapidity of contamination of the flies and the localization of the organism within the individual insects following exposure to a contaminated environment has not been previously demonstrated. Transmission via the fly vector appears to be a somewhat more complex situation, requiring more than just the fly walking across a counter or landing on exposed foodstuffs. As contaminated flies administered to hens will initiate an infection, residing on or in a fly does not eliminate the infectivity of the *Salmonella* serovar Enteritidis organism. Although we did not observe direct transmission by releasing infected flies into a room with healthy birds, the number of flies used in the challenge was modest and probably below the threshold levels necessary to result in effective transmission of *Salmonella* serovar Enteritidis from flies to poultry. Additional work is needed with higher fly challenge densities and to determine the relative roles of fly ingestion and contamination of foods by fly fecal and vomit deposits. The mechanics and parameters of pathogen spread by flies still require a more in-depth analysis before this "simple" phenomenon is understood.

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REFERENCES

1. **Ash, N., and B. Greenberg.** 1980. Vector potential of the German cockroach (Dictyoptera: Blattellidae) in dissemination of *Salmonella enteritidis* serotype *typhimurium*. J. Med. Entomol. **17:**417–423.
2. **Bailey, J. S., N. J. Stern, P. Fedorka-Cray, S. E. Craven, N. A. Cox, D. E. Cosby, S. Ladely, and M. T. Musgrove.** 2001. Sources and movement of *Salmonella* through integrated poultry operations: a multistate epidemiological investigation. J. Food Prot. **64:**1690–1697.
3. **Barrow, P. A., J. M. Simpson, and M. A. Lovell.** 1988. Intestinal colonisation in the chicken by food-poisoning *Salmonella* serotypes; microbial characteristics associated with faecal excretion. Avian Pathol. **17:**571–588.
4. **Calibeo-Hayes, D., S. S. Denning, S. M. Stringham, J. S. Guy, L. G. Smith, and D. W. Watson.** 2003. Mechanical transmission of turkey coronavirus by domestic houseflies (Musca domestica Linnaeaus). Avian Dis. **47:**149–153.
5. **Chambers, J. R., J.-R. Bisaillon, Y. Labbe, C. Poppe, and C. F. Langford.** 1998. *Salmonella* prevalence in crops of Ontario and Quebec broiler chickens at slaughter. Poult. Sci. **77:**1497–1501.
6. **Cogan, T. A., and T. J. Humphrey.** 2003. The rise and fall of *Salmonella* Enteritidis in the UK. J. Appl. Microbiol. **94:**114S–119S.
7. **De Jesús, A. J., A. R. Olsen, J. R. Bryce, and R. C. Whiting.** 2004. Quantitative contamination and transfer of *Escherichia coli* from foods by houseflies, *Musca domestica* L. (Diptera: Muscidae). Int. J. Food Microbiol. **93:** 259–262.
8. **Greenberg, B.** 1971. Flies and disease. *In* Ecology, classification and biotic associations, vol. 1. Princeton University Press, Princeton, NJ.
9. **Greenberg, B., J. A. Kowalski, and M. J. Klowden.** 1970. Factors affecting the transmission of *Salmonella* by flies: natural resistance to colonization and bacterial interference. Infect. Immun. **6:**800–809.
10. **Hald, B., H. Skovgärd, D. D. Bang, K. Pedersen, J. Dybdahl, J. B. Jespersen, and M. Madsen.** 2004. Flies and *Campylobacter* infection of broiler flocks. Emerg. Infect. Dis. **10:**1490–1492.

11. **Hargis, B. M., D. J. Caldwell, R. L. Brewer, D. E. Corrier, and J. R. DeLoach.** 1995. Evaluation of the chicken crop as a source of *Salmonella* contamination for broiler carcasses. Poult. Sci. **74:**1548–1552.
12. **Hawley, J. E., L. R. Penner, S. E. Wedberg, and W. Kulp.** 1951. The role of the house fly, Musca domestica, in the multiplication of certain enteric bacteria. Am. J. Trop. Med. Hyg. **31:**572–582.
13. **Henzler, D. J., D. C. Kradel, and W. M. Sischo.** 1998. Management and environmental risk factors for *Salmonella enteritidis* contamination of eggs. Am. J. Vet. Res. **59:**824–829.
14. **Henzler, D. J., and H. M. Opitz.** 1992. The role of mice in the epizootiology of Salmonella enteritidis infection on chicken layer farms. Avian Dis. **36:** 625–631.
15. **Holt, P. S.** 1992. Effects of induced moulting on immune responses of hens. Br. Poult. Sci. **33:**165–175.
16. **Holt, P. S.** 1992. Effect of induced molting on B cell and CT4 and CT8 T cell numbers in spleens and peripheral blood of white leghorn hens. Poult. Sci. **71:**2027–2034.
17. **Holt, P. S.** 1993. Effect of induced molting on the susceptibility of white leghorn hens to a Salmonella enteritidis infection. Avian Dis. **37:**412–417.
18. **Holt, P. S.** 1995. Horizontal transmission of Salmonella enteritidis in molted and unmolted laying chickens. Avian Dis. **39:**239–249.
19. **Holt, P. S., R. J. Buhr, D. L. Cunningham, and R. E. Porter, Jr.** 1994. Effect of two different molting procedures on a *Salmonella enteritidis* infection. Poult. Sci. **73**:1267–1275.
20. **Holt, P. S., N. P. Macri, and R. E. Porter, Jr.** 1995. Microbiological analysis of the early Salmonella enteritidis infection in molted and unmolted hens. Avian Dis. **39**:55–63.
21. **Holt, P. S., and R. E. Porter, Jr.** 1992. Microbiological and histopathological effects of an induced molt fasting procedure on a Salmonella enteritidis infection in chickens. Avian Dis. **36**:610–618.
22. **Holt, P. S., L. E. Vaughn, R. K. Gast, and H. D. Stone.** 2002. Development of a lavage procedure to collect crop secretions from live chickens for studying crop immunity. Avian Pathol. **31:**589–592.
23. **Holt, P. S., L. E. Vaughn, R. W. Moore, and R. K. Gast.** 2006. Comparison of Salmonella enterica serovar enteritidis levels in crops of fed or fasted infected hens. Avian Dis. **50:**425–429.
24. **Kopanic, R. J., B. W. Sheldon, and C. G. Wright.** 1994. Cockroaches as vectors of *Salmonella*: laboratory and field trials. J. Food Prot. **57:**125–132.
25. **Krabisch, V. P., and P. Dorn.** 1980. The importance of living vectors for the dissemination of Salmonellae in broiler flocks. Berl. Munch. Tierarztl. Wschr. **93:**232–235.
26. **Machin, J.** 1980. Cuticle water relations: towards a new cuticle water-proofing model, p. 79-103. *In* M. Locke and D. S. Smith (ed.), Insect biology in the future. Academic Press, New York, NY.
27. **Mian, L. S., H. Maag, and J. V. Tacal.** 2002. Isolation of *Salmonella* from muscoid flies at commercial animal establishments in San Bernardino County, California. J. Vector Ecol. **27:**82–85.
28. **Olsen, A. R.** 1998. Regulatory action for filth and other extraneous material. III. Review of flies and foodborne enteric disease. Reg. Toxicol. Pharmacol. **28:**199–211.
29. **Olsen, A. R., and T. S. Hammack.** 2000. Isolation of *Salmonella* spp. from the housefly, *Musca domestica* L., and the dump fly, *Hydrotaea aenescens* (Wiedemann) (Diptera: Muscidae), at caged-layer houses. J. Food Prot. **63:**958–960.
30. **Patrick, M. E., P. M. Adcock, T. M. Gomez, S. F. Altekruse, B. H. Holland, R. V. Tauxe, and D. L. Swerdlow.** 2004. *Salmonella* enteritidis infections, United States, 1985–1999. Emerg. Infect. Dis. **10:**1–7.
31. **Rosef, O., and G. Kapperud.** 1983. House flies (*Musca domestica*) as possible vectors of *Campylobacter fetus* subsp. *jejuni*. Appl. Environ. Microbiol. **45:** 381–383.
32. **Shane, S. M., M. S. Montrose, and K. S. Harrington.** 1985. Transmission of Campylobacter jejuni by the house fly (Musca domestica). Avian Dis. **29:** 384–391.
33. **Skov, M. N., A. G. Spencer, B. Hald, L. Petersen, B. Nauerby, B. Carstensen, and M. Madsen.** 2004. The role of litter beetles as potential reservoir for Salmonella enterica and thermophilic Campylobacter spp. between broiler flocks. Avian Dis. **48**:9–18.
34. **Tauxe, R. V.** 2001. *Salmonella*: a postmodern pathogen. J. Food Prot. **54:** 563–568.
35. **United Egg Producers.** 2006. Animal husbandry guidelines for U.S. egg laying flocks. http://www.uepcertified.com/docs/2006_UEPanimal_welfare_guidelines.pdf.
36. **Uyttendaele, M. R., J. M. Debevere, R. M. Lips, and K. D. Neyts.** 1998. Prevalence of *Salmonella* in poultry carcasses and their products in Belgium. Int. J. Food Microbiol. **40:**1–8.
37. **Wales, A., M. Breslin, and R. Davies.** 2006. Assessment of cleaning and disinfection in *Salmonella*-contaminated poultry layer houses using qualitative and semi-quantitative culture techniques. Vet. Microbiol. **116:**283–293.